Assessment of the direct and indirect effects of MPP+ and dopamine on the human proteasome: implications for Parkinson's Disease aetiology.

Journal:	*Journal of Neurochemistry*
Manuscript ID:	JNC-E-2007-0401.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Keywords:	20S proteasome, MPP+, dopamine, SH-SY5Y, Parkinson's disease, dysfunction

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Abbreviations: PD, Parkinson's Disease; CLA, chymotrypsin-like activity; TLA, trypsin-like activity; PLA, post-acidic-like activity; UPS, ubiquitin proteasomal system; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine

Abstract

Mitochondrial impairment, glutathione depletion and oxidative stress have been implicated in the pathogenesis of Parkinson's disease, linked recently to proteasomal dysfunction. Our study analysed how these factors influence the various activities of the proteasome in human SH-SY5Y neuroblastoma cells treated with the PD mimetics MPP^+ (a complex 1 inhibitor) or dopamine. Treatment with these toxins led to dose and time dependent reductions in ATP and glutathione and also chymotrypsin-like and post-acidic like activities; trypsin-like activity was unaffected.

Anti-oxidants blocked the effects of dopamine, but not MPP^+, suggesting that oxidative stress was more important in the dopamine-mediated effects. With MPP^+, ATP depletion was a pre-requisite for loss of proteasomal activity. Thus in a dopaminergic neuron with complex 1 dysfunction both oxidative stress and ATP depletion will contribute independently to loss of proteasomal function.

We show for the first time that addition of MPP^+ or dopamine to purified samples of the human 20S proteasome also reduced proteasomal activities; with dopamine being most damaging. As with toxin- treated cells, chymotrypsin-like activity was most sensitive and trypsin-like activity the least sensitive. The observed differential sensitivity of the various proteasomal activities to PD mimetics is novel and its significance needs further study in human cells.

Running Title: Parkinson's Disease Mimetics on proteasomeal activity.

Keywords: 20S proteasome, MPP^+, dopamine, SH-SY5Y, Parkinson's disease, dysfunction.

Introduction

Parkinson's Disease (PD) is one of the most common age-related neurodegenerative disease affecting around 1 % of the population aged over 65 years. The pathological hallmark is selective degeneration of the dopamine-containing neurons of the substantia nigra pars compacta, accompanied by the intracytoplasmic accumulation of Lewy body inclusions containing aggregated, nitrated, oxidised and ubiquitinated proteins (Forno, 1996; Good *et al.* 1998; Kowall *et al.* 2000; Lopiano *et al.* 2000; McNaught and Olanow 2006). The proteins present include α-synuclein, neurofilaments, ubiquitin, and proteasomal subunits.

In sporadic cases of PD, nigral pathology is associated with mitochondrial dysfunction, notably a reduced activity of complex 1 of the electron transport chain (Schapira *et al.* 1990; McNaught *et al.* 2001; Orth and Schapira 2002). Indeed, agents which inhibit complex 1, such as 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, produce selective degeneration of dopaminergic neurons in animal models (Beal 2001). MPTP is oxidised by monoamine oxidase to the potent neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), which selectively accumulates in dopaminergic neurons via the dopamine uptake transporter and is occasionally associated with the formation of intraneuronal inclusions reminiscent of Lewy bodies (Kowall *et al.* 2000). Although the specificity of rotenone has been questioned (Perier *et al.* 2003), chronic rotenone treatments, both in vivo and in vitro, have been reported to lead to the formation of insoluble aggregates of synuclein (Betarbet *et al.* 2000; Sherer *et al.* 2002). Although rotenone does not concentrate in dopaminergic neurons, it induces selective dopamine cell death, suggesting that these neurons are uniquely sensitive to complex 1 impairments; this may be linked to dopamine itself (see below).

Oxidative stress, accompanied by depletion of the antioxidant glutathione, is also thought to contribute to the disease (Gu *et al.* 1998; Riederer *et al.* 1989). Reactive oxygen species (ROS) may be produced during oxidative phosphorylation; within complex 1, upstream of the MPP^+/rotenone binding site, which is a site of electron leakage that enhances ROS formation (Hensley *et al.* 1998). In addition, dopamine metabolism is another source of ROS (Cassarino *et al.* 1998) and dopamine is implicated in the etiology of PD irrespective of its role as a neurotransmitter. Selective degeneration of dopaminergic neurons with MPTP/MPP^+ is reported to trigger a rapid efflux of dopamine from intracellular vesicles (Santiago *et al*., 1995), exacerbating oxidative stress. Normal catabolism of dopamine via the flavoprotein monoamine oxidase produces hydrogen peroxide (Maker *et al.* 1981). In addition, dopamine can autooxidise (Graham *et al.* 1978) and form reactive quinone and semi-quinone species that are capable of modifying cellular macromolecules, both by autooxidation and following catalysis by various enzymes (such as tyrosinase, cyclooxygenase or peroxidase) and metals (such as manganese and iron, Stokes *et al.* 2000). Under normal conditions mitochondrial hydrogen peroxide is mainly removed by its enzymatic conversion to water by glutathione peroxidase (mitochondria lack catalase). If cytoplasmic dopamine levels increase, or if cytoplasmic levels are normally high, glutathione will have a very important role in the detoxification process. Indeed, dopamine has been associated with inhibition of respiration, possibly relating to the effects of its oxidation products (Gluck *et al.* 2002), although earlier work regarding the effects of dopamine on mitochondrial respiration was contradictory (Blum *et al.* 2001; Gluck *et al.* 2002).

Recent evidence suggests that failure of the ubiquitin proteasome system (UPS) contributes to degeneration of dopaminergic neurons and Lewy body formation in sporadic PD (McNaught *et al.* 2001; McNaught and Jenner 2001). This system plays an essential role in the degradation and clearance of short-lived, mutant, misfolded or damaged proteins in eukaryotes (Goldberg, 1995). The 26S proteasome is a multi-catalytic protease which contains a catalytic 20S core, comprised of outer heptameric rings of α subunits and two inner heptameric rings of catalytically active β subunits. Some of the 20S sub-complex is associated in an ATP dependent manner with a 19S regulatory particle, making up the 26S complex, which degrades ubiquitinated proteins in an ATP-dependent process. The 19S complex utilizes ATP hydrolysis to control access of substrate to the 20S proteasome core. The best characterized proteolytic activities of the 20S complex are known as chymotrypsin-like, trypsin-like and post-acidic like (Ferrell *et al.* 2000).

Impaired protein clearance in the substantia nigra pars compacta is supported by evidence of oxidised protein accumulation (Yoritaka *et al.* 1996; McNaught *et al.* 2003) and by reduced 20S proteasomal activity in nigral post-mortem samples (McNaught *et al.* 2003). The discovery of some rare familial cases linked to genetic mutations lends further support to a failure in the UPS. Thus in autosomal dominant PD mutations in the gene coding for α-synuclein, one of the major components of Lewy bodies, have been reported (Polymeropoulos *et al.* 1997; McNaught *et al.* 2003). These mutations have been thought to cause the protein to misfold and aggregate, impairing its degradation by the UPS and inhibiting proteasomal activity (Bennett *et al.* 1999), although other mechanisms have also been proposed (Tofaris *et al.* 2001; Tofaris *et al.* 2003). In addition, loss of function mutations in two UPS enzymes, parkin (which has E3

ubiquitin ligase activity) and ubiquitin carboxy terminal hydrolase-L1, are associated with neurodegeneration in some familial forms of PD (reviewed by Farrer 2006).

The association between mitochondrial dysfunction and the proteasomal system in PD has recently been a focus of interest (Hoglinger *et al.* 2003; Shamoto-Nagai *et al.* 2003; Duke *et al.* 2006). For example, Hoglinger *et al* (2003) studied the effects of complex 1 inhibition on the survival of primary rat mesencephalic cultures, and attempted to correlate this with proteasomal activity and ATP levels. Although this work has provided very useful information there has been little or no effort to distinguish between the direct and indirect effects of the neurotoxins on proteasomal activity, and the short time courses used may have overlooked the chronic effects. The work of Hoglinger *et al* (2003) was also complicated by the use of primary, non-human (rat) embryonic cell cultures which are, by their nature, a mixed population. In addition, no attempt has been made to correlate the changes with alterations in glutathione levels, which are reportedly reduced in PD brains (Jenner, 1993) and low levels of which can disrupt the ubiquitin proteasomal pathway (Jha et al., 2002). Thus in this study the direct effects of MPP^+, MPTP and dopamine on the activity of purified human 20S proteasome are compared with the effects of the toxins on 20S proteasomal activities of human cells exposed to the neurotoxins. Human neuroblastoma SH-SY5Y cells, chosen for this study, are a widely used dopaminergic cell model for PD studies since they contain dopamine (Xavier-Gimenez et al., 2006), monoamine oxidase-A (Jiang et al., 2006) and a dopamine re-uptake system (Jiang et al., 2004), all characteristics of the dopaminergic neurons that are selectively lost in PD. The association between ATP, cellular glutathione, ROS and proteasomal activity in this model are reported in this manuscript.

Materials and Methods.

Materials

The source of materials are listed below: Tissue culture plastic ware, Scientific Laboratory Supplies (Nottingham, UK); ATP ViaLight® Plus Cell BioAssay kit, Cambrex Bioscience Ltd (Wokingham, UK); Human erythrocyte 20S proteasomes, Biomol International LP (Exeter, UK); MPP^+, dopamine and L-Buthionine-[S,R]-sulfoximine (BSO) Sigma Aldrich Chemical Co (Poole, UK). The fluorogenic peptide substrates succinyl-leucine-leucine-valine-tyrosine-7-amido-4-methylcoumarin (Suc-LLVY-AMC) and boc-leucine-arginine-arginine-7-amido-4-methylcoumarin (Boc-LRR-AMC) were purchased from Bachem Ltd (St. Helens, UK). The florogenic peptide substrate Z-leucine-leucine-Glutamic acid-7-amido-4-methylcoumarin (Z-LLE-AMC), recombinant calpain-2 enzyme (#208718) and Calpain Inhibitor III (MDL 28170) were purchased from Calbiochem (Nottingham, UK). All other chemicals were purchased from Sigma Aldrich Chemical Co. (Poole, UK) unless otherwise stated.

Cell culture

SH-SY5Y human neuroblastoma cells (from the European Collection of Animal and Cell Cultures) were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM)/Ham´s F12 (1:1) containing 10 % (v/v) heat inactivated foetal bovine serum, 1 % (v/v) MEM non-essential amino acids, 2mM L-glutamine, 200 units/ml penicillin, 20 µg/ml streptomycin (Cambrex Bioscience Ltd, Wokingham, UK) at 37°C in a humidified atmosphere of 95 % (v/v) air/ 5 % (v/v) CO_2 until 70-90 % confluent. Cells were used between passages 16-20.

Protein estimation

Protein estimation of samples was carried out using the BioRad protein assay (BioRad Laboratories Ltd, Hemel Hempstead, UK) in accordance with the manufacturer's instructions using bovine serum albumin as a standard.

Viability studies

SH-SY5Y cells (10,000 cells per well) were cultured in 96-well culture plates in growth medium in the presence or absence of either BSO (2 mM), MPP^+ (100 μM, 2 mM) or dopamine (100 μM, 2 mM) for 24 h, 48 h and 72 h. Cell viability was assessed at each time point using the MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoulium bromide; assay (Cookson et al., 1995). Briefly, 1 hour prior to the end of each incubation MTT (0.5 mg/ml, final conc) was added to each well and plates re-incubated at 37^{o}C for 1 hour. Medium was carefully aspirated from the wells and the endogenous formazan product eluted using 100 μl dimethyl sulphoxide. Plates were gently agitated to fully dissolve the formazan product, prior to reading the absorbance at 570 nm. Blank values were subtracted from each reading and the absorbance then expressed as a percentage of the value for the corresponding control.

Determination of ATP levels

SH-SY5Y cells (10,000 cells/well) were cultured in 96 well plates in growth medium in the presence or absence of BSO (2 mM), MPP^+ (100 μM, 2 mM) or dopamine (100 μM, 2 mM) for 24 h, 48 h and 72 h. ATP levels were measured using the ViaLight® Plus Cell BioAssay kit which utilizes luciferase to catalyse the formation of light from ATP and luciferin. The emitted light intensity was measured using a FLUOstar Optima luminometer (BMG Labtech, UK). Samples were protein estimated using the

BioRad assay and results were calculated as ATP levels/μg protein and expressed as % ATP levels in comparison to control samples.

Proteolytic activities

SH-SY5Y cells were plated out in 6-well plates at a density of 500,000 cells/well. Cells were exposed to either BSO, MPP^+, MPTP or dopamine for 24 h, 48 h or 72 h. After the required exposure period cells were detached using a cell scraper, centrifuged and washed with DMEM. Cell pellets were resuspended in 250 μl ice-cold homogenisation buffer [20 mM Tris/HCl, pH 7.2; 0.1 mM EDTA; 1 mM 2-mercaptoethanol; 5 mM ATP; 20 % (v/v) glycerol; 0.4 % (v/v) Igepal CA-630]. Samples were vigorously vortex mixed and cleared of cell debris by centrifugation, prior to assessment of proteasome activity. Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC were used to assess chymotrypsin-like (CLA), trypsin-like (TLA) and post acidic-like (PLA) activities, respectively (Kisselev & Goldberg, 2005). The fluorogenic peptide assay was performed using 20-30 μg of SH-SY5Y cell lysate, in a final volume of 200 μl assay buffer (50 mM HEPES pH 8.0, 5 mM EGTA). Fluorescence (360nm excitation; 465 nm emission) was measured every 5 minutes up to 2 h, linear rates calculated as ΔFU/s/μg protein and expressed as % change in comparison to control samples.

The chymotrypsin-like (CLA), trypsin-like (TLA) and post acidic-like (PLA) activities of purified human erythrocyte 20S proteasome exposed to 10 μM, 100 μM, 500 μM and 2 mM MPTP, MPP^+ and dopamine were also assessed. The fluorogenic peptide assay was performed using 0.05 μg 20S proteasome/well in a final volume of 200 μl Assay

Buffer measuring fluorescence every 5 minutes as before. The results were expressed as % change in comparison to control samples.

Activity of commercial calpain (3.6 µg) was assessed using the method described by Sasaki et al, (1984) and the substrate Suc-Leu-Tyr-AMC in a total reaction volume of 100 µl [60 mM imidazole pH 7.3, 5 mM cysteine, 2.5 µM 2-mercaptoethanol, 5 mM $CaCl_2$]. Fluoresecence was measured every minute for 20 minutes and results expressed as % change in comparison to control samples.

Analysis of total glutathione (GSH and GSSG) by enzymatic detection.

Total glutathione levels were determined using the DTNB-GSSG reductase-recycling assay, based on the method described by Anderson (1985), with minor modifications. Cells exposed to 2 mM BSO, 100 µM and 2 mM MPP^+, MPTP or dopamine for 24 h, 48 h or 72 h, were harvested by trypsinisation. Following washing the resultant cell pellet was resuspended in 200µl ice-cold sulphosalicyclic acid, sonicated for 3 x 5 seconds and centrifuged at 10,000 g for 5 minutes at 4˚C. Supernatants were then assayed for total glutathione content. Separate cell pellets were resuspended in 50 µl 1 M NaOH and protein estimated using the BioRad assay. For total glutathione analysis 140 µl NADPH (0.33 mM) and 20 µl DTNB (6 mM) were added to 20 µl aliquots of sample supernatants, GSH standards (20–4000 pmol GSH), or blanks prior to the addition of 10 µl of glutathione reductase (0.1 U/ml). Samples were made up to 200 µl with assay buffer (143 mM sodium phosphate and 6.3 mM sodium EDTA, pH 7.5) and the formation of thionitrobenzoic acid was monitored by measuring absorbance at 412 nm over 10 min. Concentrations of total glutathione (pmol/µg protein) were calculated from the GSH standard curve and expressed as % total glutathione content compared to controls.

Evaluation of ROS

SH-SY5Y cells were grown in Lab-Tek (NUNC, Scientific Laboratory Supplies, UK) chamber slides prior to treatment with MPP^+, dopamine or BSO for 72 hours. Levels of intracellular reactive oxygen species were measured using 2',7'-dichlorofluorescein diacetate (DCHDF) dye. Following treatment, medium was removed and replaced with 100 µM DCHDF for 30 min at 37°C to allow its conversion to dichlorofluorescein. Cell monolayers were then washed with Hank's balanced salt solution and ROS-activated fluorescence detected (excitation 488 nm, emission 515 nm) and photographed with a Leica TCS – NT confocal inverted microscope (Leica Microsystems, UK). All images were taken using the same laser power, gain and objective.

Statistical analysis

Data are expressed as the mean ± the standard error of the mean (SEM) and statistical analysis performed using a two-tailed, homoscedastic, Students t-Test with a 95 % confidence limit ($p < 0.05$) or a 99 % confidence limit ($p < 0.01$).

Results

Effects of MPP^+ and dopamine on SH-SY5Y cell viability.

Before studying the effect of toxins on proteasomal activity, concentrations of the toxins that were highly and mildly cytotoxic to mitotic SH-SY5Y cells over a 72 h period were established using an MTT reduction assay. This assay is dependent on cell integrity and viability and is, more specifically, a measure of metabolic activity. Figure 1 shows that MPP^+ and dopamine reduced cell viability in a dose dependent manner. Cell viability was not reduced until 48 h exposure with 100 μM MPP^+ (29 % reduction cf. controls) whilst 2 mM MPP^+ reduced cell viability by 51 % after 24 h. Dopamine at 100 μM did not affect cell viability until 72 h, reducing viability by 30 %, whilst a 500 μM dose led to a 64 % decrease in cell viability after 24h, similar to 2 mM MPP^+. Cell death did not always increase dramatically during the time course, probably due to compensatory metabolic mechanisms that occur when cells are stressed (Cookson et al., 1995). BSO toxicity was also assessed. Although, 2 mM BSO led to a 90 % reduction in glutathione levels as early as 24 h (see Fig. 6), cell viability was not affected until 96 h exposure where viability was reduced by approximately 50 % (data not shown).

In subsequent experiments, 100 μM MPP^+ and 100 μM dopamine were selected for use in SH-SY5Y cells as a mildly toxic dose (~20-30 % reduction in cell viability after 72 h) and 2 mM MPP^+ and 500 μM dopamine as a highly toxic dose (~60-70 % cell viability reduction cf. controls after 72 h).

Specificity of the different proteasomal substrates and inhibitors.

Before monitoring the effects of mildly and highly cytotoxic levels of MPP^+ and dopamine on proteasomal activity in SH-SY5Y cells, we next checked the specificity of the different proteasomal substrates and inhibitors in our cells in comparison to purified human erythrocyte 20S proteasomes.

The fluorogenic substrates Suc-LLVY-AMC, Boc-LLR-AMC and ZLLE-AMC were used to represent CLA, TLA and PLA, respectively. In an attempt to determine the specificity of these substrates for proteasome-mediated degradation, the effects of irreversible proteasome inhibitors (lactacystin and epoxomicin) and a calpain inhibitor (MDL 28170) on proteolytic activity was monitored. These inhibitors were directly added to purified 20S proteasome, calpain enzyme, and also control cell extracts, prior to assessment of protease activity. A summary of the inhibitory effects of these inhibitors is given in Table 1.

Lactacystin (10 μM) completely inhibited the CLA of purified 20S, inhibited TLA by 72 % and had no effect on PLA. In contrast epoxomicin (10 μM), reported to be a more specific proteasome inhibitor (Kisselev and Goldberg, 2005), completely inhibited both CLA and TLA of purified 20S and inhibited PLA by 80 %. The sensitivity of CLA in cell extracts to lactacystin was similar to its sensitivity in 20S, but TLA in cell extracts was insensitive to 10 μM lactacystin, as has been reported elsewhere for cell extracts (Kisselev & Goldberg, 2005). In cell extracts the sensitivities of CLA and PLA to epoxomicin were similar to 20S, suggesting that the substrates used for CLA and PLA were specific to the proteasome. In the case of TLA, however, epoxomicin reduced the activity by only 25 % suggesting that other proteases are cleaving the TLA substrate.

The calpain enzyme was completely inhibited by 10 μM MDL 28170. Addition of MDL 28170 to purified 20S proteasome resulted in 25% and 58% inhibition of CLA and TLA, respectively, and no inhibition of PLA. Lactacystin inhibited the activity of the calpain enzyme by around 40 % whilst epoxomicin had no effect. This confirms that epoxomicin is a more specific proteasome inhibitor than the more commonly used inhibitor, lactacystin. In cell extracts, 10 μM MDL 28170 did not inhibit PLA or CLA but inhibited TLA to a similar extent to 20S-TLA (~ 68 %). This would suggest that the TLA substrate can act as a substrate for calpain. Thus in order to estimate TLA in cell extracts the highly specific epoxomicin was used subsequently.

Effect of MPP^+ and dopamine on proteasomal CLA, PLA and TLA in SH-SY5Y cells

The effects of a 72 h exposure to mildly and highly toxic concentrations of MPP^+ and dopamine on the three proteasomal activities were initially checked. Control cell extracts exhibited all three proteolytic activities with the measured trypsin-like activity being the most active (Table 2a); this is consistent with data obtained using commercial human 20S proteasome (data not shown). To confirm whether the activity measured was proteasome-mediated, extracts were assayed in the presence or absence of epoxomicin.

Table 2a shows that mildly toxic concentrations of dopamine and MPP^+ (100 μM) had no effect on the three proteasomal activities. However, highly toxic concentrations of MPP^+ (2 mM) reduced CLA and PLA by 80-90%. Highly toxic concentrations of dopamine (500 μM) had a smaller effect, reducing CLA and PLA by approximately 50 %, whilst neither toxin reduced TLA. Due to the less specific nature of the TLA substrate, Boc-LLR-AMC, TLA which was sensitive to epoxomicin was regarded as

'true' proteasomal TLA (Table 2b). Data in Table 2b show that 'true' TLA was not reduced by low or high levels of either MPP^+ or dopamine; indeed TLA appeared to be activated by 100 μM MPP^+ and 500 μM dopamine.

Effect of MPP^+ and dopamine on CLA, ATP and glutathione levels in SH-SY5Y cells.

The possible roles of ATP and glutathione in maintaining proteasomal activity in response to MPP^+ and dopamine treatment was assessed, focusing on CLA since it is the most well characterized and was sensitive to MPP^+/dopamine treatment.

Control SH-SY5Y cells typically contained 5.5 ± 1.2 nmols of ATP and 20.8 ± 3.7 pmols of glutathione per μg of protein. Figure 2 represents a time course of CLA, ATP and glutathione levels after treatment with 100 μM and 2 mM MPP^+ in comparison to controls. MPP^+ affects CLA, ATP and glutathione levels in a dose and time-dependent manner. The first significant change with 100 μM MPP^+ was a reduction in glutathione levels (30 % at 48 h and 56 % at 72 h), followed by a small reduction (20 %) in ATP at 48/72 h. 100 μM MPP^+ had no significant effect on CLA activity at 24 h and 48 h, however it was reduced by 30 % at 72 h.

With 2 mM MPP^+ the first significant change was again a reduction in glutathione, which occurred at 24 h (31 %) increasing to 67 % and 82 % after 48 h and 72 h. CLA was also reduced earlier with 2 mM MPP^+ than with 100 μM MPP^+, being significantly reduced by 46 % at 48 h and 78 % at 72 h. As for CLA, ATP levels were also reduced by the 48 h time-point (48 % reduction cf. controls, increasing to 69 % after 72 h). It thus appears that a reduction in both glutathione and ATP are needed prior to a reduction in CLA.

The effects of 100 μM dopamine on CLA were similar to MPP^+ (30 % reduction at 72 h); in this case, however, glutathione levels were significantly increased (176 % cf. controls) at 24 h and were not reduced (by 23 %) until 72 h. ATP levels were not affected by this concentration of dopamine at any of the time points tested (Fig. 3).

With 500 μM dopamine, glutathione was again increased at 24 h (200 % in comparison to controls), but was reduced by 17 % and 77 % at 48 and 72 h respectively. ATP levels were non-detectable by 48 hours but CLA was not significantly reduced until 72 h. Thus it appears that ATP is less important for maintenance of CLA in the presence of dopamine in this system.

Assessment of the level of reactive oxygen species (ROS) in SH-SY5Y cells following toxin exposure.

To investigate the possible implication of increased ROS in MPP^+ and dopamine toxicity in our model, 2,7-dichlorodihydrofluorescein diacetate (DCHDF), a dye which is used as an indicator of peroxynitrate formation, was used. Following enzymatic or base-catalysed cleavage of the diacetate group, DCFDH is readily oxidised to the highly fluorescent product dichlorofluorescein (DHF). The formation of DHF can be monitored by confocal laser microscopy.

Cells in 8-well chamber slides were treated with 2 mM MPP^+, 100 μM dopamine or 2 mM BSO for up to 72 h. Since higher concentrations of dopamine (i.e. 500 μM and 2 mM) caused significant detachment from these chamber slides, a concentration of 100 μM had to be used for these experiments. A large increase in the level of ROS was evident when cells were exposed to dopamine, in contrast to cells exposed to MPP^+ where ROS production was low (Fig. 4). Treatment of cells with 2 mM BSO (which blocks glutathione synthesis; Fig. 6) also resulted in the formation of large amounts of

ROS. To investigate whether ROS formation could be blocked the effect of N-acetyl cysteine (NAC; at 1 mM), a precursor for glutathione, was included. Indeed, dopamine and MPP^+ treated cells incubated in the presence of NAC showed a reduction in the level of ROS formed.

Effect of anti-oxidants on proteasomal activity following toxic insult

To establish whether the decrease in proteasomal activity caused by the toxins could be partly due to oxidative stress, the effect 1 mM NAC on proteasomal activity of SH-SY5Y cells after 72 h exposure to toxic concentrations of MPP^+ and dopamine was monitored.

Figure 5A shows that NAC alone had no effect on CLA but 2 mM MPP^+ or dopamine drastically reduced CLA to 13.7 % and 0.5 % of controls, respectively. In the case of dopamine, the decrease in proteasomal activity by this highly toxic concentration (2 mM) was significantly reversed by 1 mM NAC (activity increasing from 0.5 % to 52.3 % of control). However, 1 mM NAC did not significantly protect CLA from MPP^+ toxicity. Similar results were obtained with other anti-oxidants (including ascorbic acid, data not shown). Visualization of cell morphology of cells exposed to toxins alone and cells exposed to toxins in the presence or absence of NAC also suggested that NAC could partly reverse the effects of dopamine, but not MPP^+ (Fig. 5B). Furthermore cells treated with dopamine and NAC were healthy and the autooxidation of dopamine, as evidenced by the colour of the growth medium, was prevented by NAC. These data indicate that the effects of dopamine (but not MPP^+) on cellular proteasome activity are likely to be primarily due to oxidative damage.

Glutathione depletion results in a reduction in both CLA and ATP levels in SH-SY5Y cells

To investigate whether glutathione depletion may be involved in reductions in CLA following treatment with MPP^+ and dopamine, glutathione levels were artificially depleted by 2 mM L-buthionine-[S,R]-sulfoximine (BSO), an inhibitor of γ- glutamyl cysteine synthase**,** an enzyme involved in the glutathione biosynthetic pathway.

As expected, 2 mM BSO virtually depleted glutathione levels as early as 24h (Fig. 6). ATP levels were also reduced by 20-30 % after 48 and 72 h and by 65 % after 96 h. Although CLA was reduced by around 30 % at 72 h, the reduction in CLA was only significant (> 50 %) after 96 h treatment with 2 mM BSO, suggesting that depletion of glutathione levels can eventually lead to proteasome impairment, possibly involving effects on ATP synthesis.

Effect of glutathione depletion on CLA in MPP^+- and dopamine-treated cells

As indicated earlier, glutathione depletion alone using BSO (2 mM) over 72 h had no significant effect on CLA (Fig. 6) whilst a 72 h exposure to 100 μM MPP^+ or dopamine resulted in a reduction in CLA (Figs. 2 and 3). However, when BSO was added in the presence of MPP^+ or dopamine , it caused a further significant reduction in CLA (Fig. 7).

Direct effect of MPP^+, MPTP and dopamine on CLA, TLA and PLA activities in purified 20S proteasome.

The effects of MPP^+ and dopamine on CLA in cell extracts was initially tested by addition of these toxins directly to a fluorogenic peptide assay containing 25-30 μg

cell extract. Whilst 100 μM MPP^+ and dopamine had no effect, both 2 mM MPP^+ and 2 mM dopamine significantly reduced CLA by 13 % of controls (data not shown).

The direct effects of the toxins on the three activities of purified human 20S were also tested, in order to check if (a) the different activities were sensitive to these chemicals and (b) whether the three activities were affected to the same extent. Both dopamine and MPP^+ reduced all three proteasomal activities but the sensitivity to low toxin concentrations was generally in the order of CLA>PLA >TLA (Table 3). All three activities appeared more sensitive to dopamine treatment than to MPP^+ (this is particularly evident with 500 μM MPP^+/dopamine, Table 3). In addition the effect of MPTP, which gives rise to MPP^+ via oxidative deamination, was also checked. Surprisingly, MPTP also reduced CLA and PLA (PLA being the most sensitive) but did not affect TLA.

Effect of ascorbic acid (vitamin C) and N-acetyl-cysteine (NAC) on neurotoxin–mediated reductions in purified human 20S proteasome activity.

Both Vitamin C and NAC protected CLA of the purified 20S proteasome against 2 mM MPP^+, MPTP and dopamine (Table 3) to a similar extent. Less vitamin C was needed to reverse the effects of MPP^+ and MPTP than dopamine, indicative of the stronger oxidative nature of dopamine (results not shown).

Discussion

This paper reports a study of the effects of two compounds of relevance to PD on the human proteasome; MPP^+ since it is known to reproduce some of the biochemical deficits of PD including inhibition of complex I (Forno 1996; Przedborski and Jackson-Lewis 1998), and dopamine because dopaminergic neurons are selectively lost in PD and dopamine is implicated in the disease process (Asanuma *et al.* 2003; Smythies 1999). We have addressed the relative roles of ATP and ROS in the determination of proteasome activity using human dopaminergic neuroblastoma cells, since previous work used rat cells. In particular we have concentrated on glutathione levels and have assessed all three proteolytic activities of the proteasome. By also studying the direct effects of these toxins on purified 20S activities we have been able to distinguish between the primary and secondary effects of the toxins on the proteasome.

Our results show that both complex I inhibition by MPP^+ and the presence of dopamine lead to a reduction in CLA and PLA (but not TLA) in our cells. We have also shown, for the first time that MPP^+ and dopamine added directly to purified 20S proteasome samples cause a reduction in proteasomal activity; with dopamine being more damaging and 20S-CLA most sensitive, followed by 20S-PLA and 20S-TLA. All three activities have previously been assessed in a limited number of human post-mortem PD samples (McNaught *et al.* 2003; McNaught and Jenner 2001), in rodent models treated chronically with MPTP (Fornai et al., 2005) or rotenone (Betarbet *et al.* 2006) and in rodent cell lines treated with complex 1 inhibitors (rotenone and MPP^+; Hoglinger *et al.* 2003) and 6-hydroxydopamine (Elkon *et al.* 2004). In these reports no attempt has been made to confirm the activities reported are truly

proteasomal and no differences in sensitivity between the three activities were highlighted. However, close inspection of the data would indicate that differences did exist in some instances (McNaught and Jenner 2001; Elkon *et al.* 2004; Fornai et al., 2005). A difference in sensitivities of the three activities is of great interest since the three active sites contribute differently and independently to protein breakdown (inhibition of multiple sites is required to markedly decrease proteolysis) and their relative importance varies with the substrate (Kisselev et al., 2006).

The effects of dopamine and MPP^+ on purified 20S-CLA in vitro were reversed by ascorbic acid. Since ascorbic acid may form direct complexes with test compounds, independent of its antioxidant properties (Zou et al., 2006), we confirmed that the effects of dopamine and MPP^+ were via an oxidative mechanism by showing that NAC could also attenuate the loss in 20S activity. Indeed dopamine is well known to be a strong oxidant and MPP^+ has previously been reported to cause direct oxidative damage to proteins (Joffe et al. 1998; Cassarino et al. 1999; Cappelletti et al. 2005). We find that MPTP (which can oxidise at room temperature in solution; Przedborski et al., 2001), also causes oxidative damage to 20S-CLA and 20S-PLA but has no significant effect on 20S-TLA. This supports the lower sensitivity of 20S-TLA in human cells. The nature of the inhibition of purified 20S proteasome by MPP^+, MPTP and dopamine appears to be non-competitive (K_m unaffected, V_{max} reduced; Supplementary Figure 1). The fact that the three PD mimetics used in this study could directly affect 20S activity would suggest that chemicals with this type of structure may have direct deleterious effects on the UPS in vivo, acting as non-competitive inhibitors, if their concentrations are sufficiently high. In support of this we found that

2 mM MPP^+ and 2 mM dopamine consistently reduced CLA, albeit by only 13%, when added to control cell extracts.

The fact that TLA activity was not reduced in cells and least affected in the 20S samples would suggest that the subunits involved with the catalytic aspects of TLA (namely β2, β6) or TLA regulation (α-subunits.) are less sensitive to oxidative stress, at least in human proteasomes exposed to damaging compounds/environments for relatively short time periods. Interestingly, similar direct effects of MPP^+, MPTP and dopamine were observed on the activity of pure trypsin enzyme; however, 20S-TLA was more sensitive than trypsin to dopamine (results not shown). This may indicate that some of the oxidative damage could be affecting the non-catalytic subunits which can moderate 20S-TLA.

When complex 1 in SH-SY5Y cells is inhibited by MPP^+ we find that both ATP and glutathione are depleted, but that ATP levels are likely to be the most important in determining whether or not CLA is reduced since removal of ROS via NAC does not protect against the effect. This is similar to the situation in rat primary mesencephalic cultures where ATP appeared to be reduced to a threshold level by MPP^+ and rotenone (20% of control value following a 6hr exposure) prior to generalized cell death linked with a 30-60 % reduction in UPS activity. Increasing ATP levels to approximately 40% of controls, by glucose supplementation, reversed the effect on proteasomal activities (Gluck *et al.* 2002; Hoglinger *et al.* 2003). However, in the present study ATP levels were reduced to only 80 % of controls by MPP^+ treatment before significant reductions in CLA occurred. This may suggest that human cells are more sensitive than rat cells to MPP^+ toxicity. Whilst proteasome impairment can be

mediated via ATP depletion it can also be independently influenced by ROS. Indeed, we find that glutathione depletion with BSO exacerbates the effect of MPP^+ on CLA. Betarbet *et al* (2006) showed that chronic exposure of human SK-N-MC neuroblastoma cells to rotenone reduced the UPS, an effect dependant on complex 1 inhibition, but in this case without ATP depletion. The effect was attenuated by α-tocopherol, indicating the involvement of oxidative stress with this complex 1 inhibitor. So it would appear that different complex 1 inhibitors have different effects.

Following treatment of SH-SY5Y cells with high levels of dopamine ATP depletion also occurred, presumably via complex 1 inhibition as previously shown in mitochondrial samples (Morikawa *et al.* 1996; Ben-Shachar *et al.* 2004) and in intact cells (Morikawa *et al.* 1996; Blum *et al.* 2001; Gimenez-Xavier *et al.* 2006). Indeed, in our experiments ATP levels were drastically reduced following a 24 h exposure to 500 μM dopamine. However, CLA was not affected until later time points. ATP levels were unaffected by 100 μM dopamine during a 72 h time-course, but CLA was significantly reduced. As expected dopamine was seen to produce vast amounts of ROS which could directly damage the proteasome, since the effects of dopamine on CLA were reversed by the anti-oxidant, NAC. The fact that dopamine treatment resulted in early increases in glutathione levels, as previously reported (Dringen 2000; Haque *et al.* 2003), supports the notion that the cells are responding to oxidative stress. Indeed dopamine did not lead to decrease in proteasomal activity until glutathione levels had been reduced. In addition, when dopamine was added together with BSO, CLA activity was further affected. We also found that dopamine, added at very high concentrations, could reduce 20S activity of cell extracts. Thus in the presence of dopamine, ATP levels are less important than ROS levels in the

maintenance of proteasomal activity, as is the case for cell viability in general (Blum *et al.* 2001; Morikawa *et al.* 1996).

As argued recently (Morikawa *et al.* 1996; Blum *et al.* 2001; Gimenez-Xavier *et al.* 2006) although dopamine concentrations of 100-500 μM, as used in this work, would be considered as being above normal physiological values, since extracelluar concentrations are reported to be of the order on nM in the substantia nigra and striatum, the intracellular striatum concentration is estimated to be around 70 μM, whilst the concentration in neuronal endings can be in the mM range. Thus it would seem likely that the extracellular dopamine concentrations used in the present study would lead to intracellular concentrations that are found in vivo in dopaminergic neurons with disrupted vesicles or vesicles not storing dopamine efficiently.

It has been reported that the ATP and ubiquitin dependent 26S is four times more vulnerable to oxidative stress than 20S (Reinheckel *et al.* 1998). This could be related to possible oxidative effects on the various regulatory subunits of the 26S proteasome (McNaught *et al.* 2003; Ishii *et al.* 2005). However, oxidative damage is not solely confined to the regulatory subunits since the β-subunits of the 20S are also reported to be targets in SH-SY5Y cells (Shamoto-Nagai *et al.* 2003). Whether or not the toxins lead to selective loss or damage to specific proteasome subunits is currently being studied in our system. It is entirely possible that some of the effects detected via measurement of 20S activity in our cellular homogenates occur via damage to the regulatory subunits, given that our extraction system contained ATP and would conserve at least some 26S particles (Coux *et al.* 1996). More importantly, a reduction in intracellular ATP levels (discussed above) could result in the dissociation of 26S

proteasomes into the less active 20S proteasome (Bernaroudj et al. 2003; Kisselev and Goldberg, 2005). This could therefore contribute, in part, to the reduction in the measured activity.

Thus our work add further support to the suggestion that complex 1 inhibition/defects, whether due to the effects of toxins or due to genetic predisposition, leads to selective problems in dopamine containing neurons since both oxidative stress and ATP depletion will contribute independently to loss of proteasomal function. It is likely that complex 1 inhibition in non-dopamine containing cells would be less damaging, and in this case ATP depletion rather than ROS would be the important determinant of 20S activity. It should also be noted that, since both MPP^+ and dopamine cause oxidative damage to purified 20S, they could have a significant direct effect on cellular proteasomal activity, provided their intra-cellular concentrations are high.

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SAMPLE	Inhibitor	Concentration (μM)	% Inhibition CLA	TLA	PLA
20S Proteasome	Lactacystin	10	95**	72*	0
	Epoxomicin	10	100**	98**	80**
	MDL 28170	10	25**	58**	0
SH-SY5Y cell extracts	Lactacystin	10	94**	0	0
	Epoxomicin	10	99**	25*	87*
	MDL 28170	10	0	67**	0

SAMPLE	Inhibitor	Concentration (μM)	% Inhibition Calpain Activity
Calpain	Lactacystin	10	39**
	Epoxomicin	10	0
	MDL 28170	10	98**

Table 1. Inhibitory effects of lactacystin, epoxomicin and MDL 28170 on purified 20S proteasome, calpain and proteasomal activity of SH-SY5Y cell extracts. Commercial sources of 20S proteasome (0.05 μg) and calpain (3.6 μg) and SH-SY5Y cell extracts (25 μg) were incubated in the presence of lactacystin, epoxomicin or MDL 28170, prior to the addition of substrate and the measurement of activity. Results were calculated as ΔFU/s/μg protein and expressed as % inhibition in comparison to control samples. When no significant inhibition was seen values were assigned "0 % Inhibition". Data presented are from a representative experiment where n=3. Statistical analysis was carried out using a two-tailed Student's t-test. Statistical significance was accepted when *= $p<0.05$ or ** = $p< 0.01$ cf. control.

(a)

Substrate	Treatment	Conc (mM)	Proteasomal Activity (10^2 ΔFU/s/μg)	Activity (%)	Epoxomicin (% Inhibition)
CLA	**Control**	**0**	2.89 ± 0.41	100.0	98.9**
	MPP+	**0.1**	2.38 ± 0.24	82.5	100.0**
		2	0.58 ± 0.34	20.2*	100.0**
	Dopamine	**0.1**	3.02 ± 0.72	104.5	100.0**
		0.5	1.60 ± 0.60	55.6*	100.0**
TLA	**Control**	**0**	24.5 ± 0.77	100.0	25.4*
	MPP+	**0.1**	39.8 ± 1.16	162.7**	31.3*
		2	21.2 ± 1.55	86.9	27.4*
	Dopamine	**0.1**	27.4 ± 1.18	112.2	22.9*
		0.5	28.3 ± 1.68	115.8	32.2**
PLA	**Control**	**0**	1.65 ± 0.08	100.0	87.2*
	MPP+	**0.1**	1.62 ± 0.09	98.6	89.3*
		2	0.17 ± 0.18	10.2**	63.4*
	Dopamine	**0.1**	1.65 ± 0.17	100.3	91.1*
		0.5	0.79 ± 0.09	48.2**	100.0**

(b)

Substrate	Treatment	Conc (mM)	Estimated 'true' TLA (%)
TLA	**Control**	**0**	100.0 ± 8.4
	MPP+	**0.1**	194.0 ± 3.4**
		2	93.5 ± 23.9
	Dopamine	**0.1**	99.3 ±4.3
		0.5	142.5 ± 25.9*

Table 2. Effect of MPP+ and dopamine on proteasomal activities from SH-SY5Y cells after 72h exposure. (a) Cells were treated with MPP+ and dopamine for 72 h prior to extraction in homogenisation buffer. Proteasome activity (CLA, TLA and PLA) was measured and results presented as ΔFU/s ± SEM and then converted to mean % activity (cf. control samples). To confirm the activity measured was proteasomal, sample activity was measured in the presence or absence of epoxomicin (10 μM) and the % inhibition calculated. When no significant inhibition was seen values were assigned "0 % Inhibition". The data presented are from a representative experiment with assays replicated in triplicate. Statistical analysis was carried out using a Student's t-test comparing toxin treated samples to their corresponding control. Statistical significance was accepted when *= $p<0.05$ or ** = $p< 0.01$. (b) Since the measured TLA contains only a fraction of actual proteasomal TLA, 'true' TLA activities were estimated using the appropriate correction factor based on epoxomicin inhibition. Statistical analysis was carried out using a Student's t-Test comparing toxin treated samples to TLA control.

Treatment	Conc (mM)	Proteasomal Activity (% cf. control)				
		CLA			TLA	PLA
			+ AA	+ NAC		
MPP^+	0.01	85.4			92.9	88.0
	0.1	71.4**			83.3	89.9
	0.5	43.3**			69.9**	31.6**
	2.0	46.1**	$70.0^{\#\#}$	$88.5^{\#\#}$	57.6**	0.3**
MPTP	0.01	91.3			97.9	70.1**
	0.1	93.4			97.0	75.5**
	0.5	66.1**			99.6	11.8**
	2.0	27.6**	$86.4^{\#\#}$	$83.5^{\#\#}$	89.4	0.0**
Dopamine	0.01	29.0**			92.2	65.2**
	0.1	32.6**			79.4**	72.5**
	0.5	12.7**			0.0**	0.0**
	2.0	0.3**	$70.2^{\#\#}$	$40.4^{\#\#}$	0.0**	0.0**

Table 3. Effects of MPTP, MPP^+ and dopamine on purified human 20S proteasome and the protective effect of ascorbic acid and N-acetyl-cysteine.

Purified 20S proteasome (0.05 μg) was incubated in the presence or absence of MPP^+, MPTP or dopamine for 10 min, prior to measuring CLA, TLA and PLA for 1 hour. The effect of ascorbic acid (+AA; 1 mM) or N-acetyl-cysteine (+NAC; 1 mM) on CLA in the presence of 2 mM MPP^+, MPTP, or dopamine was also measured. All results are presented as mean % activity in comparison to corresponding controls. Statistical analysis was carried out using the Student's t-test comparing toxin treated samples with corresponding controls (**= $p<0.01$; n=5) and comparing toxin treated CLA activity with either ascorbic acid or NAC treated CLA activity ($^{\#\#}$ = $p<0.01$, n=4). N.B. Ascorbic acid (1 mM) and NAC (1 mM) alone had no significant effect on CLA activity.

Fig. 1 Assessment of cell viability in SH-SY5Y cells following MPP^+ or dopamine exposure. Cells were seeded in 96 well plates at a density of 10,000 cells/well. After 24 h medium was exchanged for fresh growth medium supplemented with/without MPP^+ (100 μM, 2 mM) or dopamine (100 μM, 500 μM) and incubated for a further 24, 48 or 72 h, prior to viability assessment using the MTT reduction assay. Data presented are mean cell viability + SEM from three independent experiments. Statistical analysis was carried out using the Student's t test comparing toxin treated cells with corresponding time point control, where *= $p<0.05$.

Fig. 2 Effect of MPP^+ on CLA, ATP and glutathione levels in SH-SY5Y cells. Cells were exposed to 100 μM or 2 mM MPP^+ for 24, 48 and 72 h prior to assessment of CLA, ATP or glutathione content. Values were calculated as activity/μg protein and then expressed as % activity (cf. control) ± SEM, where $n\geq3$. Statistical analysis was carried out using the Student's t test comparing cells exposed to MPP^+ against time point controls, where *= $p<0.05$.

Fig. 3 Effect of dopamine on CLA, ATP and glutathione levels in SH-SY5Y cells. Cells were exposed to 100 μM or 500 μM dopamine for 24, 48 and 72 h prior to assessment of CLA, ATP or glutathione content. Values were calculated as activity/μg protein and then expressed as % activity (cf. control) ± SEM, where $n\geq3$. Statistical analysis was carried out using the Student's t test comparing cells exposed to dopamine against time point controls, where *= $p<0.05$.

Fig. 4 Determination of the level ROS in SH-SY5Y cells following toxin insult. SH-SY5Y cells were plated out in chamber slides and treated with toxins for 72 h: control (a); 2 mM BSO (b); 2 mM MPP^+ (c); 100 μM dopamine (d). The addition of NAC (1 mM) to the incubation media was also monitored in cells exposed to 2 mM MPP^+ (e) or 100 μM dopamine (f) for 72 h. Levels of ROS were determined in the presence of 100 μM DCHDF, pre-loaded for 30 minutes prior to analysis by confocal laser scanning microscopy. Scale bar represents 100 μm.

Fig. 5 Effect of NAC on CLA and cell morphology after 72 h exposure to MPP^+ or dopamine. SH-SY5Y cells were exposed to MPP^+ (2 mM) or dopamine (2 mM) in the presence or absence of 1 mM NAC for 72 h. CLA was monitored using the fluorogenic peptide assay and results were calculated as ΔFU/s/μg and then expressed as Mean % Activity + SEM (cf. control samples) – *panel (a)*. The data shown are from a representative experiment with assays replicated three times. Statistical analysis was carried out using a Student's t-test. Cells exposed to both toxin and antioxidant were compared to cells exposed to toxin alone. Statistical significance was accepted when * = $p< 0.05$ cf. control or # =$p< 0.05$ cf. toxin control. *Panel (b)* represents photographs of cells exposed to either MPP^+ or dopamine in the presence or absence of 1 mM NAC following 72 h exposure. Images were acquired with a Nikon eclipse TS100 inverted microscope. Scale bar = 50 μm.

Fig. 6 Effect of BSO on CLA, ATP and glutathione levels in SH-SY5Y cells. Cells were exposed to 2 mM BSO for 24, 48, 72 and 96 h prior to assessment of CLA, ATP or glutathione content. Values were calculated as Activity/μg protein and then expressed as % Activity (cf. control) ± SEM, where n=3. Statistical analysis was

carried out using the Student's t test comparing cells exposed to BSO against time point controls, where * = $p< 0.05$.

Fig. 7 Effect of glutathione depletion on CLA following a 72h exposure to 100 μM MPP^+ or dopamine. SH-SY5Y cells were exposed to MPP^+ (100 μM) or dopamine (100 μM) in the presence or absence of 2 mM BSO for 72h, prior to analysis of CLA. Results were calculated as ΔFU/s/μg and then expressed as mean % Activity (cf. control) + SEM. Statistical analysis was carried out using a Student's t-test comparing samples treated with toxin (MPP^+, n=4 or dopamine, n=5) to control samples or comparing samples treated with toxin + BSO to samples treated with toxin alone. Statistical significance was accepted when * = $p< 0.05$ cf. control or # = $p<0.05$ cf. toxin control.

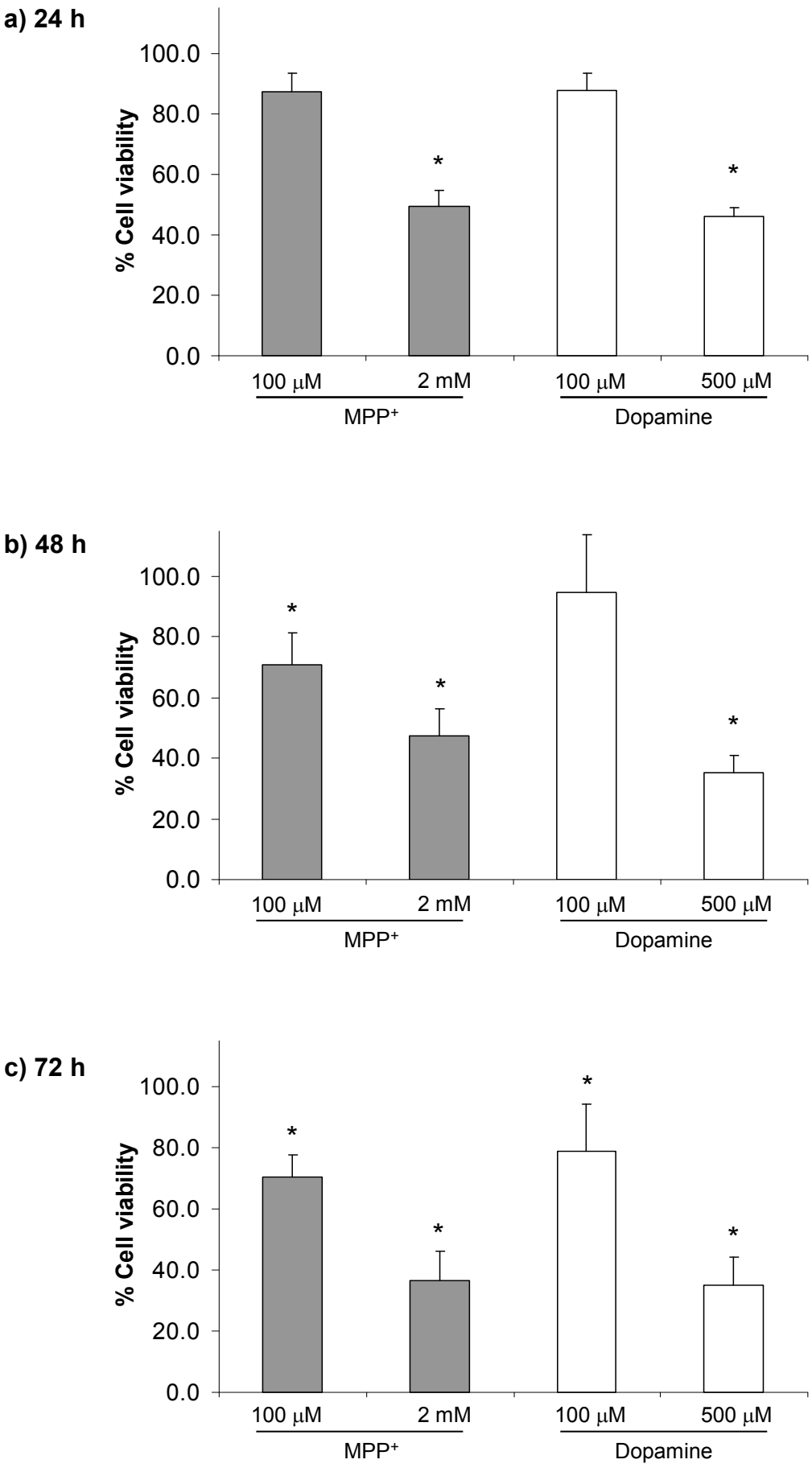

a) 24 h
b) 48 h
c) 72 h
% Cell viability
100.0
80.0
60.0
40.0
20.0
0.0
*
100 μM
2 mM
100 μM
500 μM
MPP+
Dopamine

Figure 1

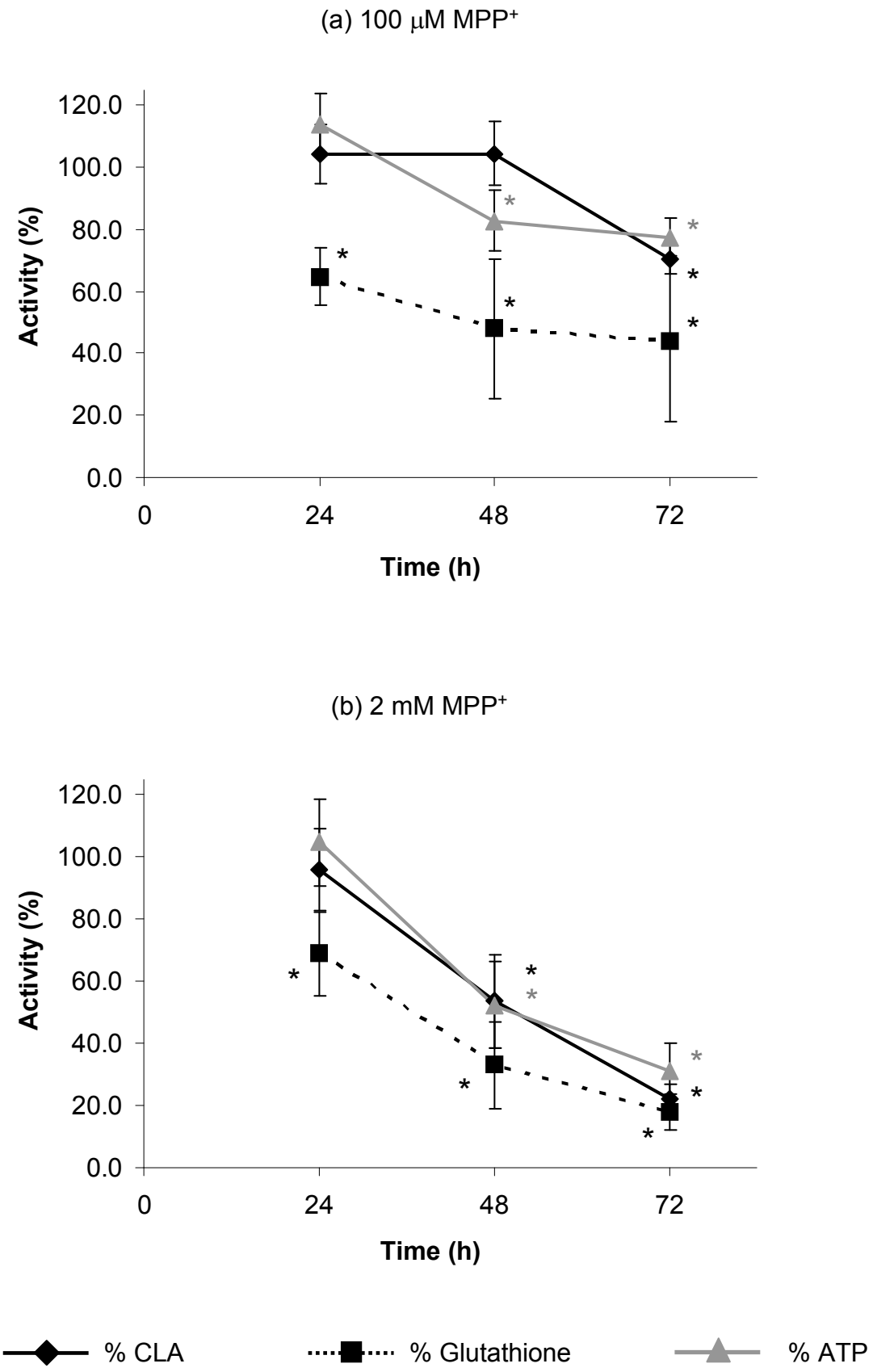

(a) 100 μM MPP+
Activity (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
24
48
72
Time (h)
(b) 2 mM MPP+
Activity (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
24
48
72
Time (h)
% CLA
% Glutathione
% ATP

Figure 2

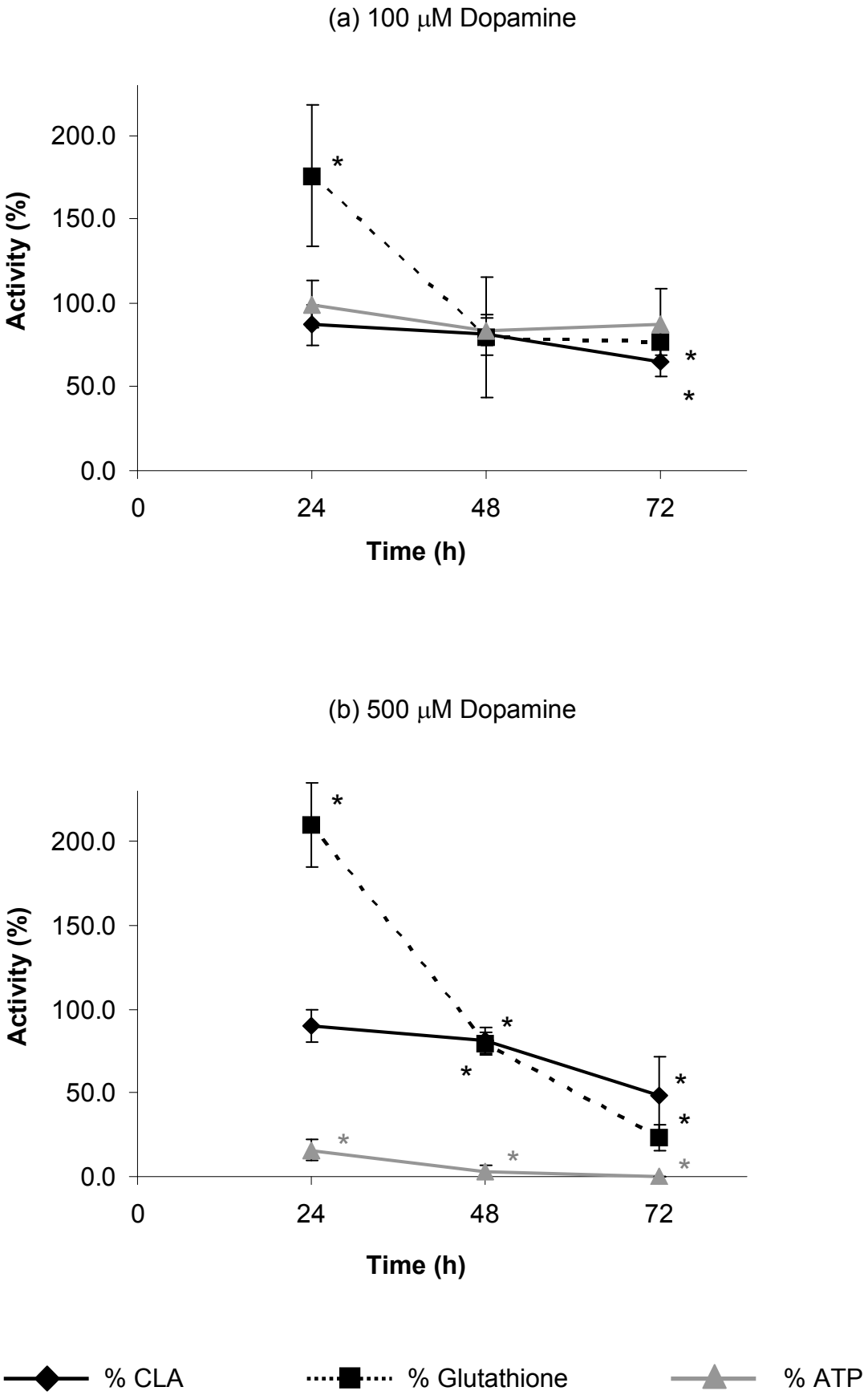
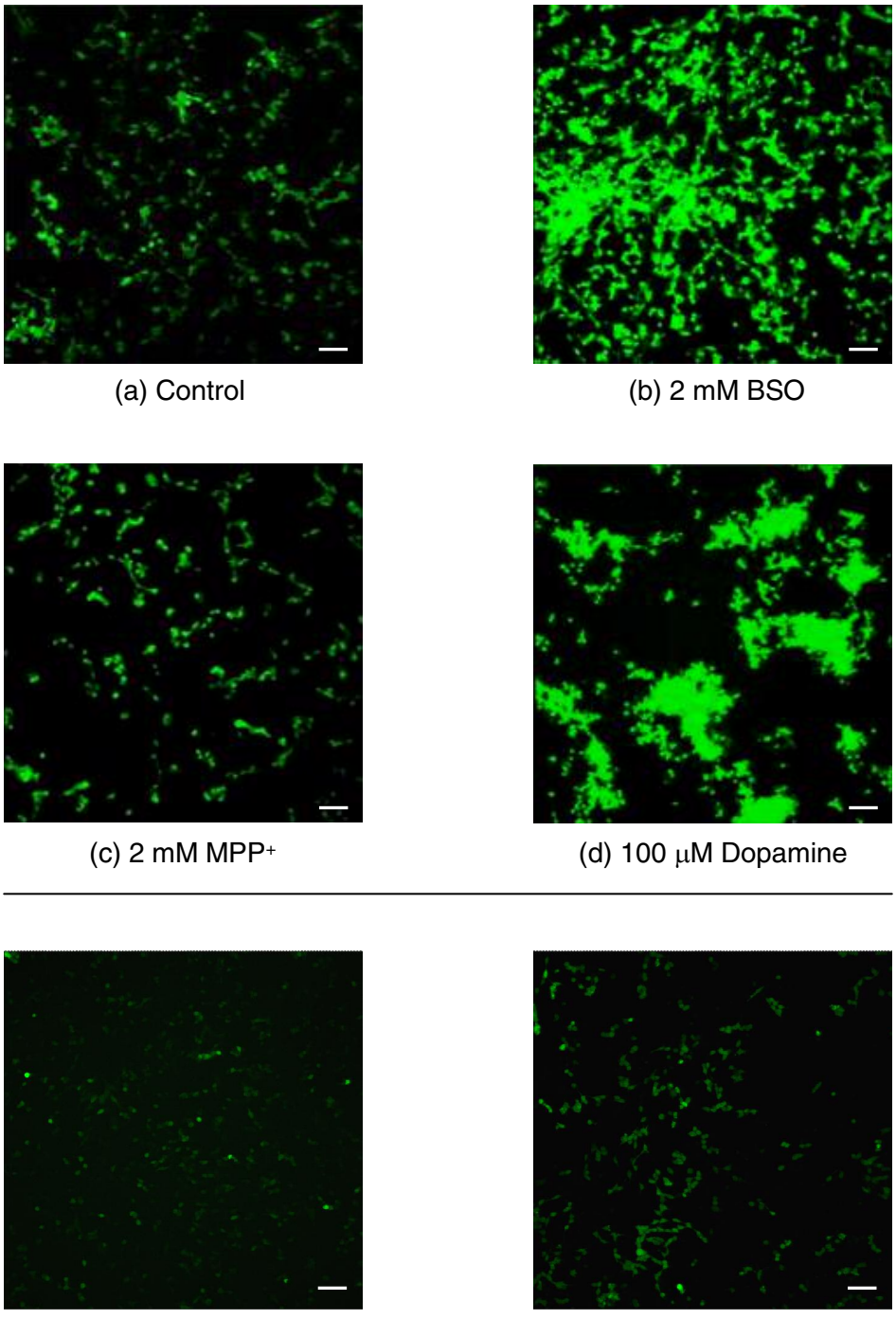

(a) 100 μM Dopamine
Activity (%)
200.0
150.0
100.0
50.0
0.0
0
24
48
72
Time (h)
(b) 500 μM Dopamine
Activity (%)
200.0
150.0
100.0
50.0
0.0
0
24
48
72
Time (h)
% CLA
% Glutathione
% ATP

Figure 3



(a) Control

(b) 2 mM BSO

(c) 2 mM MPP^+

(d) 100 μM Dopamine

(e) 2 mM MPP^+ + NAC

(f) 100 μM Dopamine + NAC

Figure 4

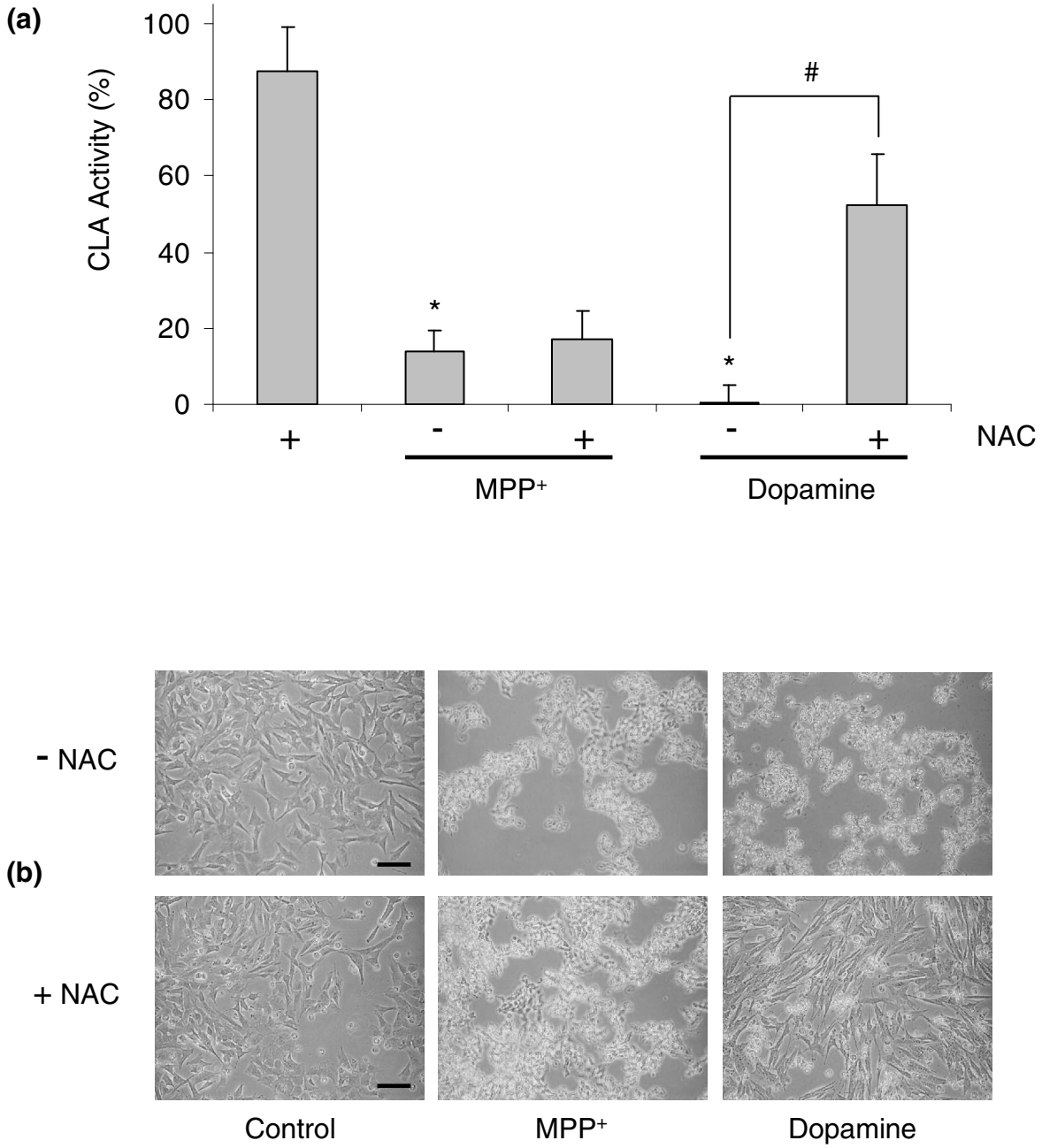

(a)
CLA Activity (%)
100
80
60
40
20
0
*
*
#
+
-
+
-
+
NAC
MPP+
Dopamine
- NAC
(b)
+ NAC
Control
MPP+
Dopamine

Figure 5

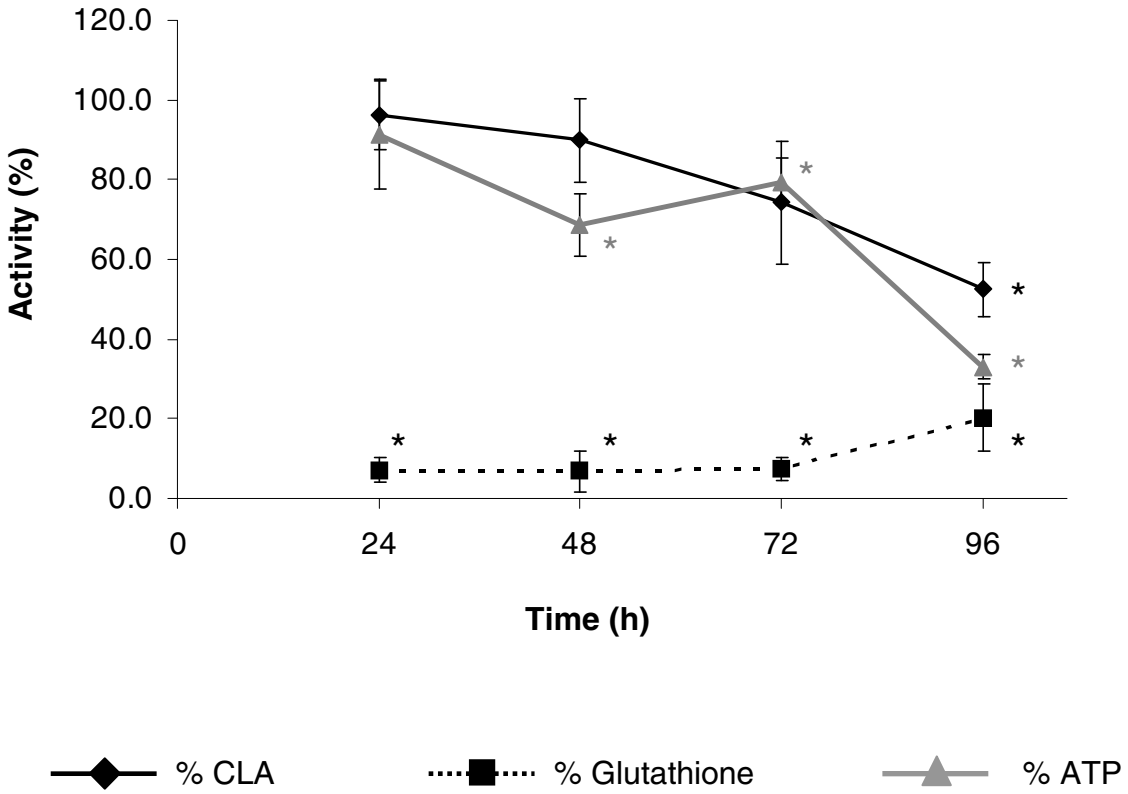

120.0
100.0
80.0
60.0
40.0
20.0
0.0
Activity (%)
0
24
48
72
96
Time (h)
% CLA
% Glutathione
% ATP

Figure 6

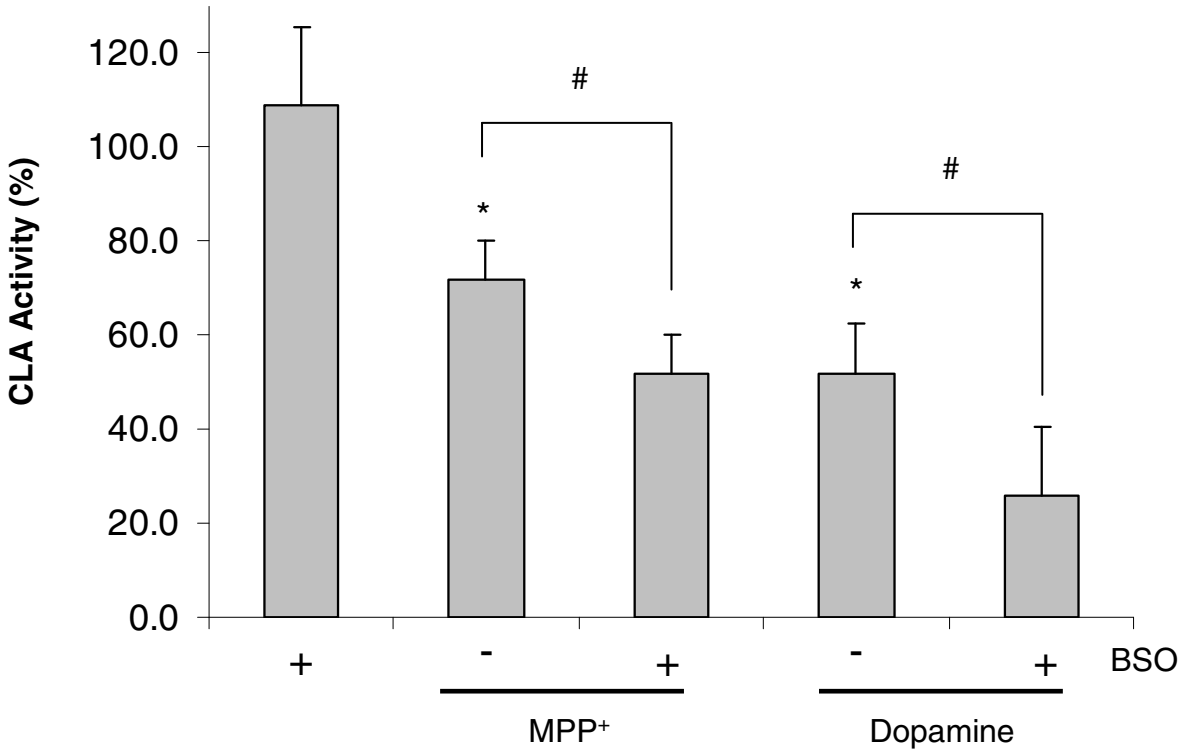
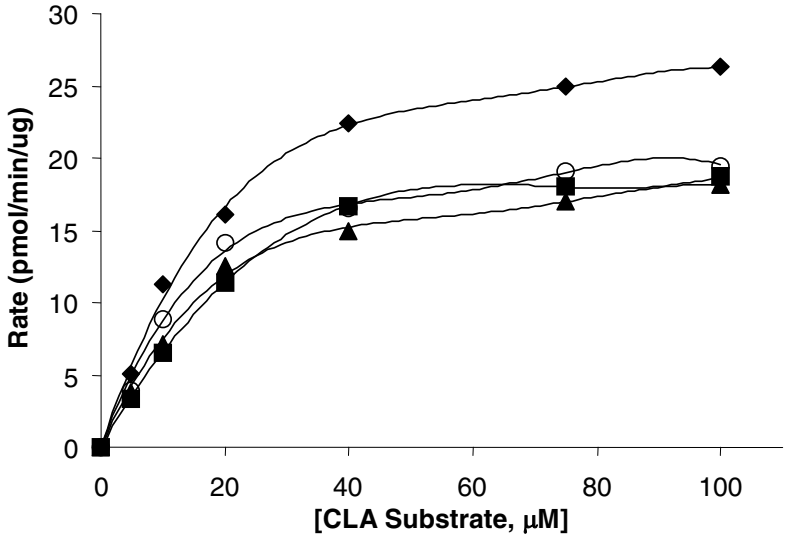
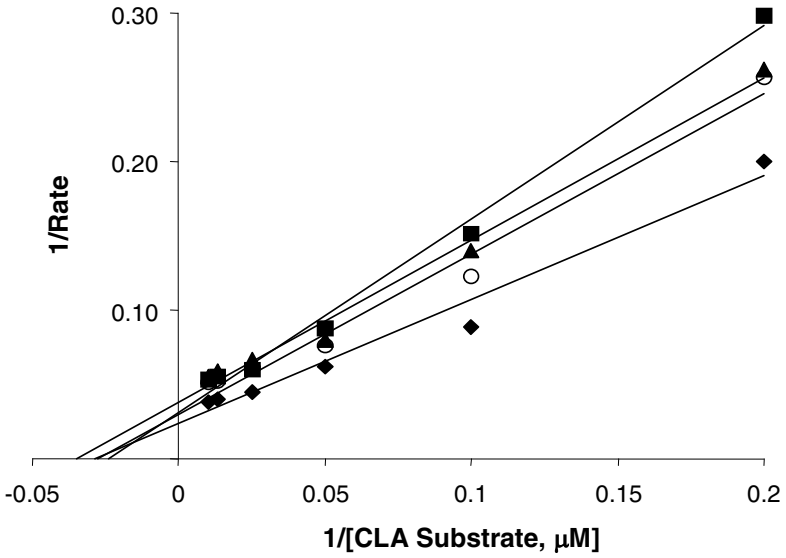


Figure 7

(a)



(b)



(c)

Treatment	k_m (μM ± SEM)	V_{max} (pmoles/min/μg ± SEM)
Control	41.22 ± 7.60	29.49 ± 3.40
500 μM MPP^+	44.06 ± 9.67	21.66 ± 4.28*
500 μM MPTP	26.63 ± 4.51	18.39 ± 2.54*
100 μM Dopamine	36.65 ± 7.91	15.47 ± 3.91*

Supplementary Figure 1. Kinetic analysis of the inhibitory effects of MPP^+, MPTP and dopamine on the chymotrypsin-like activity of the 20S proteasome. Purified 20S proteasome (0.05 μg) was incubated in the absence (control, ♦) or presence of 500 μM MPP^+ (■), 500 μM MPTP (▲) or 100 μM dopamine (○) for 10 min, prior to measuring CLA for up to 2 hours. Shown are:
(a) typical Michaelis-Menten kinetics; data are mean values for triplicate assays,
(b) Linewever-Burke plot for data in (a)
(c) V_{max} and K_m values. Data are the mean values of four experiments performed in triplicate and analysed by the Student's t test comparing toxin treated values for V_{max} and K_m with corresponding control values.